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# Phylogenetic Position of Yeastlike Endosymbionts of Anobiid Beetles

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**The Anobiid beetles *Stegobium paniceum* and *Lasioderma serricorne* possess the intracellular yeastlike symbionts *Symbiotaphrina buchneri* and *Symbiotaphrina kochii*, respectively, in the mycetome between the foregut and midgut. The nucleotide sequences of the small-subunit rRNA-encoding genes of the symbionts were determined for phylogenetic analysis. Five group I introns were found in the small-subunit rRNA-encoding genes of *S. buchneri*, but *S. kochii* lacked introns. The phylogenetic position of both symbionts was close to the class Discomycetes, which is a paraphyletic group. The two symbionts formed a monophyletic group distinct from the other major lineages. Both appear to have parted from other filamentous fungi during the early radiation of the euascomycetes and to have later become obligatory partners of the beetles.**

Insect cells are favorable dwellings for certain microorganisms, bacteria, rickettsias, viruses, and fungi. Endosymbiosis is commonly observed with blood-sucking, plant-juice-sucking, and saprophagous insects (8). It is, however, unclear how many of these microorganisms have established mutualistic associations with host insects. To investigate this enigmatic process in the evolution of insects and microorganisms, the taxonomic and phylogenetic status of the endosymbionts, which is mostly unknown, should be elucidated. The recent development of molecular systematics has revealed that the bacterial endosymbionts of aphids, *Buchnera* spp., belong to the gamma-3 subgroup of the class *Proteobacteria* (1, 44) and the yeastlike symbionts (YLS) of planthoppers are pyrenomycetous fungi in the subphylum Ascomycotina (35).

The anobiid beetles *Stegobium paniceum* (the drugstore beetle) and *Lasioderma serricorne* (the cigarette beetle) evolved from wood-eating ancestral species; with the advent of agriculture they readily adapted to become stored-product pests. They attack many drugs, pepper, spices, seeds, and processed foods of all kinds (12, 30). The beetles harbor YLS in the cells of the cecum between the foregut and midgut. These symbionts are transmitted from generation to generation of beetles. The symbionts are smeared on the egg surface at oviposition, and newly hatched larvae eat the symbionts on the egg case. The YLS provide nutrients, such as vitamin B and sterol (36), and detoxify substances which are deleterious to the host beetle (17, 41). The YLS of *S. paniceum* and *L. serricorne* have been cultured by many researchers and are now classified as *Symbiotaphrina buchneri* (*S. paniceum* YLS [SPYLS]) and *Symbiotaphrina kochii) L. serricorne* YLS [LSYLS]), respectively, in the family Taphrinaceae (21, 46). However, knowledge of the relationship of *Symbiotaphrina* spp. to other yeasts and fungi is limited and the placement of the genus *Symbiotaphrina* in the family Taphrinaceae is not universally accepted (7, 26).

This paper reports the phylogenetic position of SPYLS and LSYLS and gives full nucleotide sequences of the small-subunit rRNA-encoding genes (SSU rDNA) from the two *Symbiotaphrina* species. The presence of multiple insertions of group I introns in the SSU rDNA of SPYLS is reported, and the size, multiple insertions, and insertion positions of the introns in SSU rDNA are also discussed in relation to the taxa related to the symbionts.

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## MATERIALS AND METHODS

**Insects and YLS.** The two species of anobiid beetles (order Coleoptera, family Anobiidae), *S. paniceum* (Linnaeus) and *L. serricorne* (Fabricius), were provided by H. Fujii of the National Food Research Institute and were reared on wheat bran. The alimentary tracts from adult beetles were aseptically dissected, and YLS from the gut ceca were isolated on agar plates (34). Saline homogenates of the ceca were spread on YPD agar (0.5% yeast extract, 1% peptone, 2% glucose, and 1.5% agar) or PPGA (potato extract [20%, wt/final vol], 0.5% peptone, 0.5% glucose, 0.3% $Na_2HPO_4$-$12H_2O$, 0.05% $KH_2PO_4$, and 1.5% agar) and incubated at 26°C. For the collection of yeast cells, YLS were incubated in YPD liquid medium at 28°C and centrifuged at 600 × *g* for 5 min. *Saccharomyces cerevisiae* (strain FR1870) was provided by H. Takano and A. Hino of the National Food Research Institute.

**DNA preparation and PCR amplification.** Cultured YLS were suspended in 50 mM EDTA, pH 8.0. One-third of a volume of Lyticase (Sigma) solution (0.2% in 0.01 M sodium phosphate buffer, pH 7.5, containing 50% glycerol) was added, and the YLS suspension was incubated at 37°C for 30 min to make YLS spheroplasts. Extraction of nucleic acid from the spheroplasts was carried out by the yeast DNA miniprep method described by Rose et al. (39).

The following primers designed by White et al. (47) were used to amplify a part of or nearly full-sized SSU rDNA. Their sequences and positions on SSU rRNA of *S. cerevisiae* (29) are as follows: NS1, 5′-GTAGTCATATGCTTGTCTC-3′ (positions 20 to 38); NS2, 5′-GGCTGCTGGCACCAGACTTGC-3′ (positions 573 to 553); NS4, 5′-CTTCCGTCAATTCCTTTAAG-3′ (positions 1150 to 1131); NS5, 5′-AACTTAAAGGAATTGACGGAAG-3′ (positions 1129 to 1150); NS6, 5′-GCATCACAGACCTGTTATTGCCTC-3′ (positions 1436 to 1413); NS7, 5′-GAGGCAATAACAGGTCTGTGATGC-3′ (positions 1413 to 1436); NS8, 5′-TCCGCAGGTTCACCTACGGA-3′ (positions 1788 to 1769). Forward primers are indicated by odd numbers, and reverse primers are indicated by even numbers. The primers were synthesized by a DNA synthesizer (model 392; Applied Biosystems). Conditions for PCR were as described previously (35).

Terminal sequences of SSU rDNA, those at upstream flanking regions of primers NS1 and at downstream flanking regions of NS8, were not amplified by these primers. To obtain both the terminal sequences of the SSU rDNA, inverse PCR (2) was performed with the above-described primers and two new primers, SP1, 5′-CAGCTCGCAGCTGAGTACTACG-3′ (the sequence corresponding to a part of a intervening sequence beside the NS8 primer region in SSU rDNA of SPYLS), and SP2, 5′-GAGCCATTCGCAGTTTCACAGT-3′ (corresponding to positions 99 to 78 in SSU rDNA of *S. cerevisiae*). Extracted DNA (500 ng) was treated with an endonuclease for 60 min. To determine the sequences of the 5′ and 3′ ends of SPYLS and the 5′ and 3′ ends of LSYLS, endonucleases *Pst*I, *Pst*I, *Eco*RI, and *Xba*I were used, respectively. The amplified PCR products were ligated with a DNA ligation kit (TAKARA) at 16°C for 60 min to make the DNA circular, and the terminal sequences of SSU rDNA were amplified by combinations of the aforementioned primers, NS5-SP2, SP1-NS6, NS7-SP2, and NS7-

TABLE 1. Ascomycetes used for the phylogenetic analysis and their DNA database accession numbers

| Ascomycete | Accession no. |
|---|---|
| Plectomycetes | |
| *A. apis* (Maasen ex Claussen) Olive et Spiltoir | M83264 |
| *M. purpureus* Went | M83260 |
| *T. flavus* (Klocker) Stolk et Samson | M83262 |
| Pyrenomycetes | |
| *H. chrysospermus* Tul | M89993 |
| *O. ulmi* (Buisman) Nannfeldt | M83261 |
| *P. anserina* (Cesati) Niess | X54864 |
| Loculoascomycetes | |
| *L. doliolum* (Fr.) de Not | U04205 |
| *S. nodorum* Berkeley | U04236 |
| Discomycetes | |
| Caliciales | |
| *M. albonigrum* (W. Nylander) L. Tibell | L37538 |
| *S. globosus* (Huds.) Vain | L37532 |
| Lecanorales | |
| *L. dispersa* (Pers.) Sommerf | L37535 |
| *P. crustulata* (Ach.) Hertel et Knoph | L37540 |
| Leotiales | |
| *L. lubrica* (Scop.) Pers.:Fr. | L37536 |
| *S. sclerotiorum* (Libert) de Bary | X69850 |
| *S. flavida* Pers.:Fr. | Z30239 |
| Pezizales | |
| *A. lineolatus* Van Brummelen | L37533 |
| *I. aggregata* (Berk. et Br.) Surček | Z30241 |
| *M. elata* Fr. | L37537 |
| *P. badia* Pers.:Fr. | L37539 |
| *P. nigrella* (Pers.:Fr.) Karst. | Z27408 |
| Hemiascomycetes | |
| *C. albicans* (Robin) Berkhout | X53497 |
| *S. cerevisiae* Meyen ex Hansen | J01353 |
| Archiascomycetes | |
| *S. complicata* S. Goto, J. Sugiyama, M. Hamamoto et K. Komagata | D12530 |
| *T. deformans* (Fuckel) Tulasne | U00971 |
| *S. buchneri* Gräbner ex W. Gams et von Arx | D94657 |
| *S. kochii* Jurzitza ex W. Grams et von Arx | D94656 |
| Ascomycetous yeast | |
| *A. pullulans* (de Bary) Arnaud | M55639 |

NS2, for the 5′ and 3′ ends of SPYLS and the 5′ and 3′ ends of LSYLS, respectively.

To examine the sequences of mature rRNA of SPYLS, the rRNA was reverse transcribed and then subjected to PCR (reverse transcription-PCR). The extracted nucleic acids, which included DNA and RNA, were used as templates. cDNA was synthesized by using the NS4 or NS8 primer, and the two cDNA products were amplified by conventional PCR using NS1 and NS4 primers or NS5 and NS8 primers, respectively. cDNA synthesis was performed in 20 μl of the buffer used for the PCR (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 0.001% gelatin) with 0.5 mM each deoxynucleoside triphosphate, 100 pmol of primer, RNA (approximately 300 ng), 1 U of RNase inhibitor (TOYOBO), and 26 U of reverse transcriptase (avian myeloblastosis virus reverse transcriptase; TAKARA). The reaction mixture was incubated at 42°C for 60 min, and 2 μl of the mixture was used for the PCR amplification.

**Cloning and sequencing.** Amplified PCR products were treated with a DNA-blunting kit (TAKARA) to make the termini of DNAs blunt. The DNA was passed through a minicolumn (60 by 7.5 mm; about 2.5 ml) of Sepharose CL-2B (Pharmacia LKB) and was then inserted into the *Sma*I or *Eco*RV site of pBluescript II (Stratagene).

Plasmid DNA was isolated by the alkaline lysis procedure (40) and was used for sequencing templates. The sequences from the two clones were determined in opposite orientations. The insert DNAs were unidirectionally deleted by exonuclease digestion (DNA deletion kit; TAKARA), and the sequences of the deletion mutants were determined by the *Taq* dye primer cycle sequencing method with a DNA Sequence System (model 373A; Applied Biosystems).

**Phylogenetic analysis.** Twenty-five ascomycetes were selected for phylogenetic analysis (Table 1). *Sclerotinia sclerotiorum* (X69850) had a group I intron in its SSU rDNA; therefore, the intron was removed from the sequences for the purpose of analysis. The five group I introns in the SSU rDNA of SPYLS were also deleted from the sequence for the analysis. Stretches of about 1,570 bp of the SSU rDNA sequences of 27 species, including SPYLS and LSYLS, from positions 114 to 1680 in *S. cerevisiae*, were aligned by using CLUSTAL V software (distributed by D. G. Higgins, European Molecular Biology Laboratory) for multiple sequence alignment. Gaps in the alignment data were manually deleted from the sequences, and 1,466 nucleotides (nt) were used for cladistic analysis. Tree topology was built by the neighbor-joining method in PHYLIP (Phylogeny Inference Package) version 3.5C (distributed by J. Felsenstein, University of Washington). Confidence values for individual branches of the resulting tree were determined by a bootstrap analysis in which 500 bootstrap trees were generated from resampled data.

**Accession numbers of symbionts.** The *Symbiotaphrina* species used in this study, SPYLS (= SANK 50595 = JCM 9740 = IFO 10845) and LSYLS (= SANK 50495 = JCM 9739 = IFO 10846), have been deposited with Sankyo Research Laboratories, Sankyo Co., Ltd., Tsukuba, Japan (SANK); the Japan Collection of Microorganisms, Institute of Physical and Chemical Research (Riken), Wako, Japan (JCM); and Institute Fermentation, Osaka, Japan (IFO).

**Nucleotide sequence accession numbers.** The GSDB, DDBJ, EMBL, and NCBI accession numbers for the nucleotide sequences of the SSU rDNAs of LSYLS and SPYLS are D49656 and D49657, respectively.

## RESULTS

**Culture of the symbionts.** Colonies of SPYLS were cream to pale pinkish red and mucoid. The cells were round and 3 to 5 μm long. Colonies of LSYLS were cream and mucoid. The cells were round, oval, or pyriform, 2 to 3 μm wide, and 3 to 4.5 μm long. The budding cells of both symbionts showed enteroblastic basipetal conidiation at the attenuated end. They did not form ascospores, basidiospores, and mycelia. Glucose was not fermented, and the diazonium blue B test gave a negative result. These characteristics of SPYLS and LSYLS are the same as those described previously for the two organisms (21, 46).

**Nuclear SSU rDNA of anobiid beetles.** Nuclear SSU rDNAs from SPYLS and LSYLS were amplified by PCR with a pair of primers, NS1 and NS8, which were able to amplify nearly full-sized SSU rDNA from *S. cerevisiae* except for 19 bases at the 5′ terminus and 10 bases at the 3′ terminus. The size of the product from LSYLS was similar to the size of that from *S. cerevisiae*, at about 1,800 bp, which is a usual size for fungal SSU rDNA. However, a single large product, of about 2,900 bp (Fig. 1, lane 9), was amplified from SPYLS. Parts of the SSU rDNA of SPYLS were then amplified with another pair of primers. With NS1 and NS2, NS5 and NS6, and NS7 and NS8,

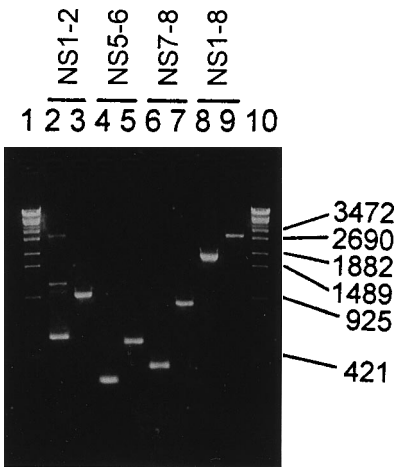


FIG. 1. Agarose gel electrophoresis of PCR products from *S. cerevisiae* (lanes 2, 4, 6, and 8) and SPYLS (lanes 3, 5, 7, and 9) SSU rDNA. rDNA was amplified by using a combination of a forward primer and a reverse primer. Lanes: 2 and 3, primers NS1 and NS2; 4 and 5, primers NS5 and NS6; 6 and 7, primers NS7 and NS8; 8 and 9, primers NS1 and NS8; 1 and 10, DNA markers (lambda-*Eco*T14I digest; TAKARA). Numbers on the right indicate size in base pairs.

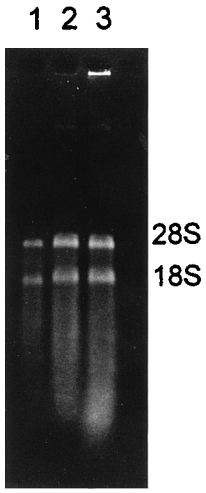


FIG. 2. Mature SSU rRNA of LSYLS (lane 2) and SPYLS (lane 3). Lane 1, large-subunit (28S) and SSU (18S) rRNA of *S. cerevisiae* (Pharmacia LKB).

the sizes of SPYLS amplification products were about 950, 500, and 800 bp (Fig. 1, lanes 3, 5, and 7), respectively; those of *S. cerevisiae* were about 550, 300, and 400 bp (lanes 2, 4, and 6), respectively.

The amplified PCR products were cloned in a plasmid and then sequenced. The flanking sequences outside the NS1 and NS8 primers in the SSU rDNA of SPYLS and LSYLS were obtained by inverse PCR. rDNAs from both SPYLS and LSYLS had the same flanking (terminal) sequences as did that from *S. cerevisiae* (29). Therefore, both of the SSU rDNA termini from SPYLS and LSYLS were regarded as being identical to those from *S. cerevisiae*. The sizes of the rDNAs of SPYLS and LSYLS were 2,879 and 1,801 bp, respectively. The rDNA of SPYLS had five inserted sequences which were not found in the rDNA of LSYLS. The sizes of the five insertions were 194, 210, 210, 232, and 232 bp, in order of the insertion position from the 5′ end of the gene. In the present paper, these insertions are called SPYLS SSU1 to SSU5, respectively. Without the insertions, the rDNA of SPYLS was highly homologous to that of LSYLS, with a total of only 7 of 1,801 bases differing.

The SSU rRNA of SPYLS was similar in size to those of LSYLS and *S. cerevisiae* (Fig. 2); this suggests that the mature SSU rRNA does not include the insertions. This was confirmed by sequence analysis of cDNA which was synthesized from the rRNA by reverse transcription-PCR. The five insertions were completely deleted from the mature rRNA. The five insertions are likely to come after a thymidine (T) in the rDNA exon sequences and end with a guanine (G). This is a characteristic of group I introns in boundary sequences (10); i.e., RNA sequences contain conserved splice site nucleotides, U at the putative 5′ splice site and G at the putative 3′ splice site. The insertion sites of the group I introns, SSU1 to SSU5, were at nt 98 (between nt 98 and 99), 550, 1676, 2055, and 2616 of the SSU rDNA sequence of SPYLS, respectively. These positions correspond to nt 98, 356, 1272, 1441, and 1770, respectively, in LSYLS SSU rDNA. All five introns were inserted in the conserved region of the rRNA (31).

The putative secondary structures of the introns (data not shown) indicated that all five introns lack P5 extensions and P7.1 or P7.2 insertions (9). Internal guide sequences, which are proposed to align the splice sites for cleavage and exon ligation (13), were also found in the introns and flanking exon sequences.

**Phylogeny of the symbionts.** Despite the presence of the introns, the nucleotide sequence of the SSU rDNA of SPYLS was quite similar to that of LSYLS. Therefore, by using the nucleotide sequence of the SSU rDNA of LSYLS, a homology search of DNA database libraries was performed by using the FASTA family program (37). The homology search selected some ascomycetous fungi, including *Aureobasidium pullulans*. Preliminary analysis with SSU rDNA indicated that the two symbionts were closely related to discomycetous fungi. Subsequently, 25 ascomycetes, mainly members of the class Discomycetes, were selected for the cladistic analysis.

The phylogenetic tree of the ascomycetes (Fig. 3) supports the evolutionary relationship, which is well established from recent studies based on the nucleotide sequences of SSU rDNA (5, 48). Archiascomycetes (33) (*Saitoella complicata* and *Taphrina deformans*) and hemiascomycetes (*S. cerevisiae* and *Candida albicans*) showed lineages different from those of euascomycetes. Pyrenomycetes (*Hypomyces chrysospermus*, *Podospora anserina*, and *Ophiostoma ulmi*), plectomycetes (*Talaromyces flavus*, *Monascus purpureus*, and *Ascosphaera apis*), and loculoascomycetes (*Septoria nodorum* and *Leptoshaeria doliolum*) formed separate lineages. The tree suggests that the above-described major lineages of euascomycetes radiated in a relatively short period of time in evolutionary terms. The class Discomycetes, as pointed out by Gargas and Taylor (23), is a paraphyletic assemblage. Among these apothecial ascomycetes, members of the order Pezizales (*Plectania nigrella*, *Inermisia aggregata*, *Morchella elata*, *Peziza badia*, and *Ascobolus lineolatus*) seem to have diverged first in the euascomycetes and to have formed a separate monophyletic group. *Leotia lubrica*, a member of the order Leotiales, was not grouped with other members of the same order (*Spathularia flavida* and *S. sclerotiorum*), but bootstrap percentages which support this separation of *L. lubrica* from other members of the order Leotiales were low. The tree also seems to support monophyly for two members of the order Lecanorales (*Lecanora dispersa* and *Porpidia crustulata*) together with *Sphaerophorus globosus*, which is a member of the order Caliciales. The taxonomic positions of *Mycocalicium albonigrum* and *A. pullulans* were not clear. The symbionts, LSYLS and SPYLS, clearly formed a distinct lineage. They apparently parted from other ascomycetes during the early radiation of euascomycetes (23).

## DISCUSSION

The YLS of anobiid beetles were formerly placed in the genus *Torulopsis* (24). They were later reclassified into the genus *Symbiotaphrina* (27, 45) because they were regarded as being similar to some species of *Taphrina* (26). The names *S. buchneri* and *S. kochii* were validated for the symbionts of the beetles *S. paniceum* and *L. serricorne*, respectively (21). The present phylogenetic analysis, however, indicated that the symbionts are euascomycetes and phylogenetically distant from *T. deformans*, which is a member of the class Archiascomycetes. It proved difficult to classify them by conventional morphological and culturing methods. The results show that the symbionts of beetles formed a distinct lineage after the radiation of euascomycetes. They appear to have originated from a filamentous euascomycetous ancestor and to have them lost the ability to make mycelia after taking up residence in the beetles. The results of this study indicate that the placement of the genus *Symbiotaphrina* into the family Taphrinaceae (21, 46) is probably inappropriate.

Recent molecular phylogenetic studies, based on nucleotide sequences, have resulted in the discovery of many introns in

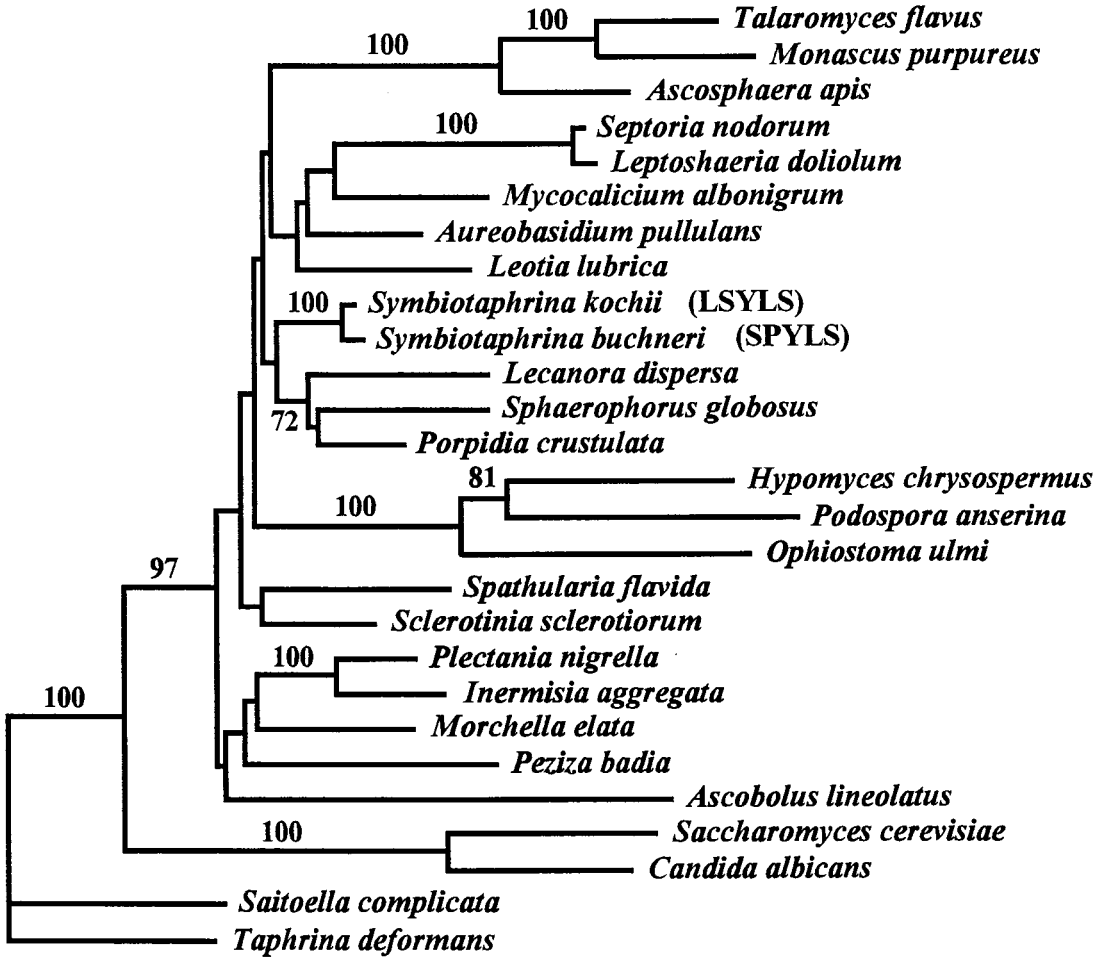


FIG. 3. Phylogenetic relationships of SPYLS and LSYLS to 25 selected species of ascomycetes. The tree was constructed by a neighbor-joining procedure with the software package PHYLIP, version 3.5C, by using nucleotide sequences of SSU rDNA. Numbers indicate bootstrap percent confidence.

nuclear rDNA, especially SSU rDNA. Group I introns show considerable differences in size, sequence, and distribution among organisms. Group I introns in nuclear SSU rDNA are found in some amoebae, algae, and fungi (the group I intron database [11]). Fungal species which have group I introns in the SSU rDNA are *Ustilago maydis* (16), *Rhodosporidium dacryoidum* (accession number D13459) (subphylum Basidiomycotina), *Cenococcum geophilum* (38) (subphylum Deuteromycotina), and the following ascomycetous fungi: *Pneumocystis carinii* (42), *Protomyces inouyei* (32) (class Archiascomycetes), *Cladonia chlorophaea* (15), *L. dispersa* (22) (lichen-forming fungi, order Lecanorales), *S. sclerotiorum* (X69850), and *Hymenoscyphus ericae* (20) (order Leotiales). The last four species are in the class Discomycetes. Gargas et al. (22) have also reported that the discomycetous fungi *Calicium tricolor*, *M. albonigrum* (order Caliciales), and *P. crustulata* (order Lecanorales) have insertions, some of which might be group I introns. This study revealed that the symbionts of anobiid beetles, which were closely related to the discomycetous fungi, also had group I introns in the SSU rDNA.

A model for the present diversity of group I introns is based on intron mobility, which has been demonstrated (18, 19). This model also suggests that group I introns were introduced into the nuclei of later-diverging species by gene transfer via intron-containing bacteria (3, 28). However, some group I introns, which lack an endonuclease coding region, appear to be nonmobile (4) and it is suggested that introns have been acquired at an early stage in evolution and lost in the majority of *Naegleria* spp. (14). Bhattacharya et al. (6) suggested that group I introns in nuclear SSU rDNA have been laterally transferred during evolution and that after transfer, some of these elements may become stable components of the host cell nuclear genome. The recently generally accepted scenario is that the modern distribution of introns is due to lateral transfer between organisms and insertion and deletion. In this connection, SPYLS from another colony of *S. paniceum*, which was collected and provided by Masahiro Sakai of Ehime University, also had long SSU rDNA and the insertion of the same five introns was deduced from the endonuclease restriction patterns of the rDNA (unpublished data). However, the type culture of *S. buchneri* (CBS 420.63) does not have any introns in about 1,200 bp of the 5′ region of the SSU rDNA (25a). With SPYLS, it is more likely that the type culture species has lost its introns in the SSU rDNA than that the five group I introns were independently transferred to SSU rDNA of SPYLS in the beetles from two different locations. It is not known when the introns disappeared from the SSU rDNA of the type culture species. Some group I introns in fungus nuclear SSU rDNA might have been inherited from ancestral species, because most of the group I introns found in nuclear

SSU rDNA do not have enzyme-encoding open reading frames and some introns present in different species are inserted at identical positions in SSU rDNA. For example, *C. geophilum* (38), *H. ericae* (20), *P. carinii* (42), *P. inouyei* (32), and SPYLS have the insertion at position 1506 (according to numbering for *Escherichia coli* SSU rDNA) and *P. inouyei* (32), *S. sclerotiorum* (X69850), and *U. maydis* (16) have the insertion at position 943. These two insertion positions were also found in the SSU rDNA of some algae (22). Gargas et al. (22) suggested that the insertion of introns at the same positions in both fungi and algae (positions 516, 943, 1046, and 1506) represents relatively ancient events.

The structures of group I introns are characterized by the presence of nine base-paired stem domains (P1 to P9) and a conserved core region consisting of four conserved elements (P, Q, R, and S) (9). The usual size of the introns is more than 300 bp (group I intron database [11]), and when the introns have an open reading frame which encodes an enzyme, they are larger. The five introns found in SPYLS were relatively small in comparison with other group I introns reported to date, at 194 to 232 bp. Among fungi, only in the SSU rDNAs of *C. chlorophaea* (210 to 228 bp) (15) and *L. dispersa* (189 to 231 bp) (22) are there introns as small as those of SPYLS. Multiple insertion of group I introns in nuclear SSU rDNA is also reported for these two species. The only other fungal species whose SSU rDNA shows multiple insertions is *P. inouyei*; this species has two insertion positions (32). These two species are members of the order Lecanorales in the class Discomycetes. *C. chlorophaea* has five different insertion positions (positions 1046, 1203, 1210, 1389, and 1516, numbered according to positions in *Escherichia coli* SSU rDNA) (15), and *L. dispersa* has eight, including a degenerated intron (positions 114, 287, 516, 789, 943, 1046, 1210, and 1516) (22). The insertions in the SSU rDNA of SPYLS were at positions 114, 287, 1052, 1210, and 1506. Insertions at position 1210 are shared by the three species, and those at positions 114 and 287 were shared by only *L. dispersa* and SPYLS among all group I intron-possessing species reported to date. As far as the small size, multiple insertions, and insertion positions 114 and 287 in group I introns of SSU rDNA are concerned, the YLS and the lichen-forming fungi, especially *L. dispersa*, are similar. The phylogenetic analysis showed that the group nearest to YLS was the order Lecanorales, which includes *L. dispersa*. It is reasonable to assume that insertions of introns in the YLS and the lichen-forming fungi occurred during relatively ancient times. The symbionts and lichen-forming fungi might have an unknown factor(s) which keeps the introns from being deleted, or many group I introns might have been introduced in the SSU rDNA after the fungi began to evolve separately from other filamentous fungi and before the separation of YLS and the order Lecanorales. The relative abundance of introns in the present discomycetous fungi is likely to be the result of the inheritance of anciently inserted introns. The results also suggest that the symbionts of anobiid beetles are closely related to the lichen-forming fungi. It is interesting that both the lichen-forming fungi and YLS have found symbiotic relationships with algae or beetles.

Berbee and Taylor (5) proposed that lineages of filamentous ascomycetes radiated from approximately 280 million (180 to 320 million) years ago. Gullan and Cranston (25) stated that the orders of modern insects appear to have been established by the beginning of the Triassic, 225 million years ago. The ancestral species of anobiid beetles, which began to harbor a filamentous ascomycete in gut cells, must have appeared at a much later date. It is usually assumed that the endosymbionts' initial association with insect hosts was as pathogenic parasites or nonpathogenic commensals (43). In the former case, the hypothesis is that the pathogens later became attenuated and began to live in the insect body and eventually inside certain cells. In the latter scenario the hypothesis is that, for example, the microorganisms were often eaten by the ancestral host insect species and had the opportunity to infect gut cells, thus developing into intracellular symbionts. With the anobiid beetles, the latter is likely to have occurred because these beetles have a mycetome in the gut tissue and are infected by ingestion of symbionts every generation. Steinhaus (43) proposed an evolutionary process whereby extracellular symbionts which had lived constantly in the lumen of the gut later began living in the mycetomes of insects. More studies of the closest relatives of the symbionts are required before we can postulate a more precise process for the development of endosymbiosis in anobiid beetles.

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